EMBO reports

scientific report

Identification of short 'eukaryotic' Okazaki fragments synthesized from a prokaryotic replication origin

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Although archaeal genomes encode proteins similar to eukaryotic replication factors, the hyperthermophilic archaeon *Pyrococcus abyssi* replicates its circular chromosome at a high rate from a single origin (*oriC*) as in Bacteria. In further elucidating the mechanism of archaeal DNA replication, we have studied the elongation step of DNA replication *in vivo*. We have detected, in two main archaeal phyla, short RNA-primed replication intermediates whose structure and length are very similar to those of eukaryotic Okazaki fragments. Mapping of replication initiation points further showed that discontinuous DNA replication in *P. abyssi* starts at a well-defined site within the *oriC* recently identified in this hyperthermophile. Short Okazaki fragments and a high replication speed imply a very efficient turnover of Okazaki fragments in Archaea. Archaea therefore have a unique replication system showing mechanistic similarities to both Bacteria and Eukarya.

EMBO reports **4**, 154–158 (2003)

doi:10.1038/sj.embor.embor732

INTRODUCTION

In Bacteria and Eukarya, bi-directional replication origins are used for the duplication of genetic material. Because both strands of the DNA duplex are replicated simultaneously and all known DNA polymerases synthesize DNA only in the 5′→3′ direction, one of the strands of the DNA duplex (the lagging strand) is replicated 'backwards' by repetitive synthesis of short RNA-primed DNA strands, named Okazaki fragments (reviewed recently by Waga & Stilmann (1998) and Benkovic *et al.* (2001)). After the removal of RNA primers and their replacement with DNA, Okazaki fragments are end-joined to form an uninterrupted DNA strand carrying genetic information (MacNeill, 2001). A transition point of continuous and discontinuous DNA synthesis on a given strand therefore marks a replication origin. Although discontinuous DNA synthesis is an analogous process in Bacteria and Eukarya, the mechanisms participating in the synthesis of the lagging strand show important differences in these two domains. For instance, for unknown reasons, the length of bacterial Okazaki fragments (1–2 kilobases (kb); Kornberg & Baker, 1992) is up to 10-fold that in Eukarya (up to 125 nucleotides; Blumenthal & Clark, 1977; Bielinsky & Gerbi, 1999). Moreover, many key enzymes, such as the primase, DNA helicase and DNA polymerases implicated in lagging-strand DNA synthesis in Bacteria and Eukarya, are not evolutionarily related. Together these observations have suggested that molecular processes for DNA replication in Bacteria and Eukarya might have independent origins (Leipe *et al.*, 1999; Forterre, 1999).

Until recently, little was known about the DNA replication mechanism in the other prokaryotic domain, Archaea. Comparative genomics have indicated that most archaeal DNA replication proteins are orthologous to eukaryotic ones (Bohlke *et al.*, 2002), thus predicting similarities in archaeal and eukaryotic replication mechanisms. Nevertheless, we have recently shown that, similarly to Bacteria, the circular chromosome of the hyperthermophilic archaeon *Pyrococcus abyssi* has a single replication origin (*oriC*; Myllykallio *et al.*, 2000). This *oriC* has several repeated sequences and A/T-rich regions, where initiator proteins Cdc6/Orc1 and minichromosome maintenance (MCM) helicase bind specifically to start bi-directional DNA replication (Matsunaga *et al.*, 2001). In addition, the replication rate in *P. abyssi* (20 kb min^{-1}; Myllykallio *et al.*, 2000) and *Sulfolobus solfataricus* (15 kb min^{-1}; Hjort & Bernander, 2001) is similar to that in Bacteria and much higher than that in Eukarya (0.5–5 kb min^{-1}; Kornberg & Baker, 1992). Archaea are thus able to replicate their genome more rapidly than Eukarya despite their use of eukaryotic-like proteins for DNA replication. The answer to whether the high replication speed in Archaea coincides with long Okazaki fragments, as in Bacteria, remained elusive, because the length of archaeal Okazaki fragments *in vivo* has not been investigated. Moreover, even the presence of RNA-primed replication intermediates in Archaea has not been evident until now, because *Pyrococcus* primase can uniquely synthesize both DNA and RNA *de novo* without the help of a pre-existing primer (Bocquier *et al.*, 2001; Liu *et al.*, 2001).

Here we report the detection of short RNA-primed replication intermediates in the hyperthermophilic archaea *P. abyssi* and *Sulfolobus acidocaldarius* by two independent methods. These short RNA-primed replication intermediates originate solely from the retrograde arm of the replication fork at the *P. abyssi oriC*, thus indicating that they must correspond to archaeal Okazaki fragments. These data have revealed the first prokaryotic genome replicated with both bacterial (a single origin and a high replication speed) and eukaryotic (homologous replication proteins and short Okazaki fragments) features. As the length and structure of eukaryotic and archaeal Okazaki fragments are very similar, our data further suggest that the frequent

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Received 5 August 2002; revised 3 September 2002; accepted 27 November 2002
Published online 24 January 2003

turnover of Okazaki fragments, and not the high processivity of lagging-strand synthesis, allows highly efficient duplication of genetic material in Archaea.

RESULTS AND DISCUSSION

RNA priming of DNA replication in Archaea

To gain insight into the mechanisms causing the drastic difference in the length of Okazaki fragments in Bacteria and Eukarya, we attempted to detect RNA-primed DNA replication intermediates in DNA samples isolated from rapidly dividing cells of *P. abyssi* (a member of the Euryarcheota containing eukaryotic-like histones) and *S. acidocaldarius* (a member of the Crenarcheota lacking histones), representing the two major phyla among Archaea. In an unmasking assay (Fig. 1) (DePamphilis, 1995), total DNA from exponentially growing cells was first phosphorylated with non-radioactive ATP to mask all free 5′-OH ends. DNA fragments containing RNA at their 5′ ends were then unmasked by alkaline hydrolysis, and their exposed 5′-OH ends were labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. In both organisms, our results indicate the presence of RNA-containing DNA fragments approximately 100 nucleotides in length that are clearly shorter than bacterial Okazaki fragments. Background signals seen in the control reaction (lanes without NaOH treatment) probably result from the exchange reaction between [γ-^{32}P]ATP and the 5′-phosphate of DNA fragments, which is known to occur inefficiently at 4 °C (DePamphilis, 1995). To confirm that these putative replication intermediates resulted from initiation *de novo* and not from strand breakage, DNA samples from *P. abyssi* were also labelled by using vaccinia guanylyltransferase, an RNA-specific capping enzyme used in the direct labelling of RNA primers of replication intermediates (DePamphilis, 1995). Because labelled products were analysed by denaturing polyacrylamide-gel electrophoresis, this experiment provides a better estimate of the size of archaeal Okazaki fragments than the unmasking assay. Similarly to the unmasking assay, the use of guanylyltransferase revealed short DNA fragments with a covalently attached RNA segment at their 5′ ends in *P. abyssi* (Fig. 2A) with a size distribution from 10 to 120 nucleotides on denaturing polyacrylamide gels. The structure of *P. abyssi* RNA/DNA fragments detected in the above experiments is thus strikingly similar to Okazaki fragments detected in *Drosophila* cells (Blumenthal & Clark, 1977) and in *Saccharomyces cerevisiae* (Bielinsky & Gerbi, 1999), demonstrating that archaeal Okazaki fragments are very similar to eukaryotic ones (Fig. 2B).

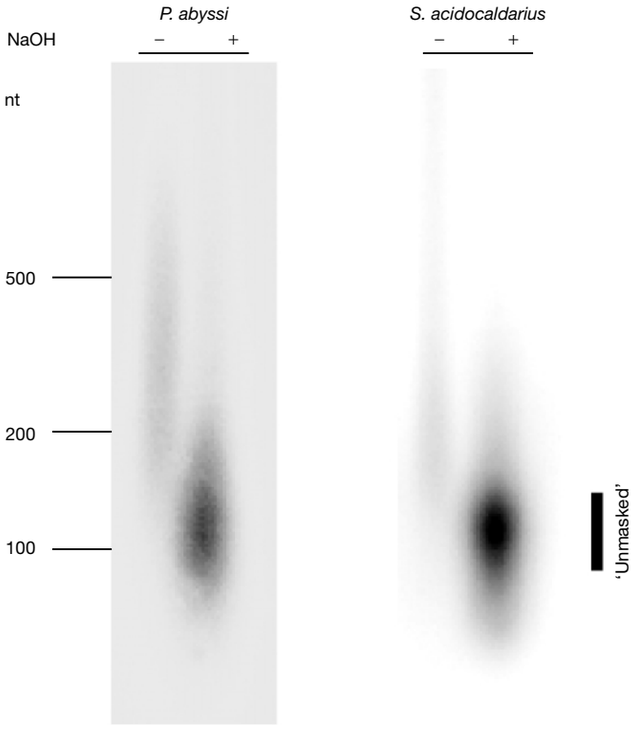

Fig. 1 | Unmasking assay showing short replication intermediates in *Pyrococcus abyssi* and *Sulfolobus acidocaldarius*. The 5′-OH groups of RNA-primed replication intermediates were selectively exposed by alkaline treatment. 'Unmasked' 5′-OH ends of replication intermediates were labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and run on an alkaline agarose gel. A control experiment without alkaline treatment is also shown for each experiment. The positions of size markers (100, 200 and 500 nucleotides (nt)) are shown on the left.

Analyses *in vitro* have indicated that the catalytic subunit of *Pyrococcus* primase (Pfup41) alone preferentially uses deoxynucleotides to synthesize DNA segments up to several kilobases in length (Bocquier *et al.*, 2001), whereas the catalytic subunit of *Methanococcus* primase synthesizes RNA primers (Desogus *et al.*, 1999). The obvious discrepancy between our results *in vivo* and the biochemical activity of Pfup41 can be explained by the recent observation (Liu *et al.*, 2001) that the primase activity of a catalytic subunit Pfup41 is drastically modulated by the accessory subunit Pfup46 (p58 in Eukarya). In particular, unlike Pfup41 alone, the Pfup41–p46 complex is capable of RNA primer synthesis that is highly stimulated by ATP. It is also noteworthy that the high intracellular salt concentration in *P. abyssi* cells (500–600 mM) helps in stabilizing the primer–template complex and the RNA itself at high temperature (Hethke *et al.*, 1999).

The detection of short Okazaki fragments together with a known replication speed in Archaea has allowed us to calculate that, on average, 2.2 and 1.6 Okazaki fragments per second are initiated during chromosomal replication in *P. abyssi* and *S. acidocaldarius*, respectively. This rate is higher than has been observed previously for Bacteria (0.4–0.8 Okazaki fragments per second) or in Eukarya (~0.2 Okazaki fragments per second), indicating that lagging-strand synthesis requires an extremely frequent turnover of Okazaki fragments in Archaea. How this high productivity in archaeal Okazaki fragment synthesis, and possibly processing, is achieved remains to be established. However, studies *in vitro* have indicated that loading of PCNA (proliferating-cell nuclear antigen), a step that is required for the synthesis of each Okazaki fragment, proceeds with a relatively high efficiency in *P. furiousus* (Cann *et al.*, 1999). Alternatively, as a functional counterpart for the eukaryotic polymerase-α is not found in Archaea, omitting the primase–polymerase-α switching process at the lagging strand could contribute to the frequent turnover of Okazaki fragments. Recent biochemical studies have indicated that in Eukarya, Dna2 and FEN1 endonucleases, together with RP-A (replication protein A), have crucial roles in Okazaki fragment processing by removing RNA and DNA segments synthesized by primase–polymerase-α (Bae & Seo, 2000; Bae *et al.*, 2001). However, several putative Dna2 endonuclease/helicases seem to be present in Archaea, complicating the functional annotation of archaeal proteins

that participate in the synthesis and processing of Okazaki fragments. It is also noteworthy that, whereas *Euryarchaeota* have histone H3 and H4 homologues, histone homologues are absent in *Crenarchaeota* (Sandman *et al.*, 2001). Despite this, practically identical size distributions of RNA-primed replication intermediates were detected for *P. abyssi* and *S. acidocaldarius* (Fig. 1), indicating that histone proteins, or the nucleosomal organization of chromatin, do not limit the length of Okazaki fragments in Archaea.

Okazaki fragments at the *P. abyssi oriC*

The identification of RNA primers in archaeal replication intermediates has permitted us to employ replication initiation point (RIP) mapping to investigate the detailed replication pattern at the *P. abyssi oriC* locus. RNA-primed DNA molecules are protected against degradation by λ-exonuclease, allowing the detection of RNA/DNA junctions in nascent DNA preparation by primer extension with radioactive primers. We therefore purified nascent DNA from *P. abyssi* cells with a combination of a benzoylated naphtholyated DEAE (BND)-cellulose column and the selective digestion of nicked DNA molecules with λ-exonuclease. Primer extension reactions performed with the obtained nascent DNA preparations, by using several primers hybridizing to two leading strands at different locations in the *oriC* (see Fig. 4A), were analysed on a sequencing gel. These experiments revealed distinct bands denoting individual DNA start sites within the analysed region (Fig. 3). The smallest fragments detected with primers F1 and R2 were longer than the replication intermediates observed by unmasking and capping assays, indicating that they denoted the leading-strand initiation sites. When the lagging strand was analysed with the primer F2, frequent initiation sites with an interval of 40–60 bases were detected. These results confirmed that replication intermediates observed by unmasking and capping assays are indeed short Okazaki fragments. Moreover, when both leading strands were analysed, we detected the presence of a well-defined transition point for each strand within this *oriC*. These transition points spanned a region of 7 bp (Fig. 4B), and this region was located just adjacent to the long inverted-repeat on the left edge of the *oriC* region shown in Fig. 4A. This inverted repeat is well conserved among the replication origins of the three *Pyrococcus* species whose genomes have been completely sequenced. The inverted repeat contained an internal sequence that matched the consensus sequence of short 13-bp repeats (Fig. 4C) that we have noticed previously in this chromosomal region. Whereas the short repeat is found in 10 different places in the *P. abyssi* genome, the long inverted-repeat in the vicinity of the transition points could not be found in the other parts of the genome (data not shown). Moreover, this long inverted-repeat surrounded a putative DNA unwinding element (DUE; Fig. 4A), which was presumably required for the unwinding of the DNA duplex during replication initiation. We have shown previously that *P. abyssi* Cdc6/Orc1 and MCM proteins bind to this chromosomal locus preferentially in exponential-phase cultures (Matsunaga *et al.*, 2001). The structure formed by this inverted repeat might therefore function as a molecular target for the initiation factors and/or elongation factors recruited at the replication origin.

In the absence of appropriate replication mutants or chemical inhibitors of DNA replication in hyperthermophilic Archaea, we cannot currently conclude whether population heterogeneity exists with regard to the selection of a start site. However, independent results from unmasking (Fig. 1), capping (Fig. 2) and RIP (Fig. 3) assays all indicate that the size distribution of archaeal Okazaki fragments is very similar to that of Eukarya. An as yet unidentified common feature between archaeal and eukaryotic replication systems must therefore determine the low processivity of lagging-strand synthesis in these two domains. Earlier results *in vitro* have indicated that *Pyrococcus* primase (p41–p46 complex) starts primer synthesis by incorporating ATP (Liu *et al.*, 2001), indicating that the template recognized by

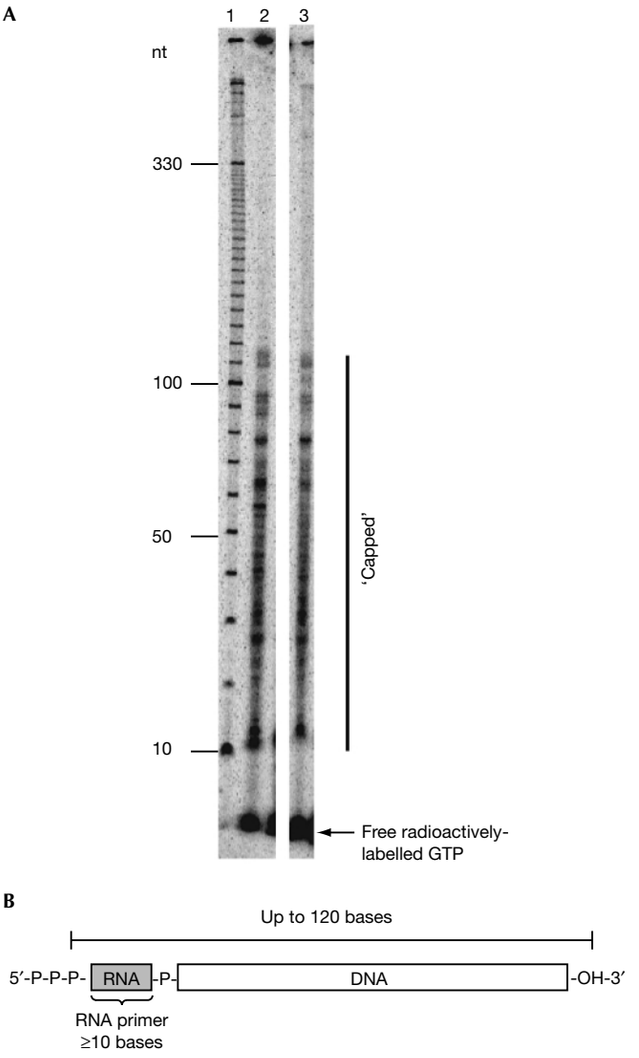

Fig. 2 | Direct labelling of RNA on the 5′ termini of replication intermediates with vaccinia virus guanylyltransferase, an enzyme responsible for messenger RNA capping. (**A**) The 5′ ends of RNA primers attached to *P. abyssi* replication intermediates were capped with radioactive GTP and samples were fractionated on a sequencing gel (lane 2). Lane 3 contains the same reaction as lane 2, except that total DNA was treated with the restriction endonuclease *Eco*RI before analysis, confirming that nascent strands were efficiently denatured from template strands. Lane 1, size marker (10-base ladder; Gibco-BRL) labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. (**B**) Schematic diagram of the archaeal Okazaki fragment. The smallest replication intermediate detected (10 nt) is in accordance with the minimum length of RNA primers detected *in vitro* (Liu *et al.*, 2001).

DNA primase contains thymidine. The development of an *in vitro* system that permits the direct sequence analysis of RNA primers is now necessary to identify this putative thymidine-containing sequence motif that is recognized by the primase complex.

CONCLUSION

We have identified short eukaryotic-like Okazaki fragments in two archaeal species (*Pyrococcus* and *Sulfolobus*) representing the two main archaeal phyla. In particular, short RNA-primed replication intermediates have been localized at the *P. abyssi* replication origin, permitting the precise mapping of the transition points between the leading and lagging strands. This will help to elucidate further the mechanism of DNA replication initiation in Archaea. Our results have also shown that the length of Okazaki fragments is not correlated with either replication speed or nucleosomal structure. Further studies of the unique archaeal replication system will require an *in vitro* system combining purified proteins and the precisely mapped origin of bi-directional replication identified in this study.

METHODS

Cell culture and DNA purification. *P. abyssi* and *S. acidocaldarius* cells were grown in VSM medium at 95 °C and in *Sulfolobus* medium at 80 °C, respectively (Lopez-Garcia & Forterre, 1999; Myllykallio *et al.*, 2000). Cells collected from the exponential phase were re-suspended in TNE buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA and 100 mM NaCl) and disrupted by the addition of 1% SDS and 1% sodium sarkosyl. After extraction with phenol:chloroform (1:1), 100 μg ml^{-1} Hoechst-33258 and 1.1 g ml^{-1} CsCl were added to the DNA solution, and the refractive index was adjusted to within the range 1.398–1.401. Total genomic DNA was purified by CsCl gradient ultracentrifugation with standard procedures. For the RIP assay, replication intermediates were further enriched by BND-cellulose chromatography. Total DNA was passed down a BND-cellulose column pre-equilibrated with NET buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 M NaCl) to selectively bind replication intermediates with single-stranded regions. After being washed extensively with NET buffer, bound DNA was eluted with NET buffer containing

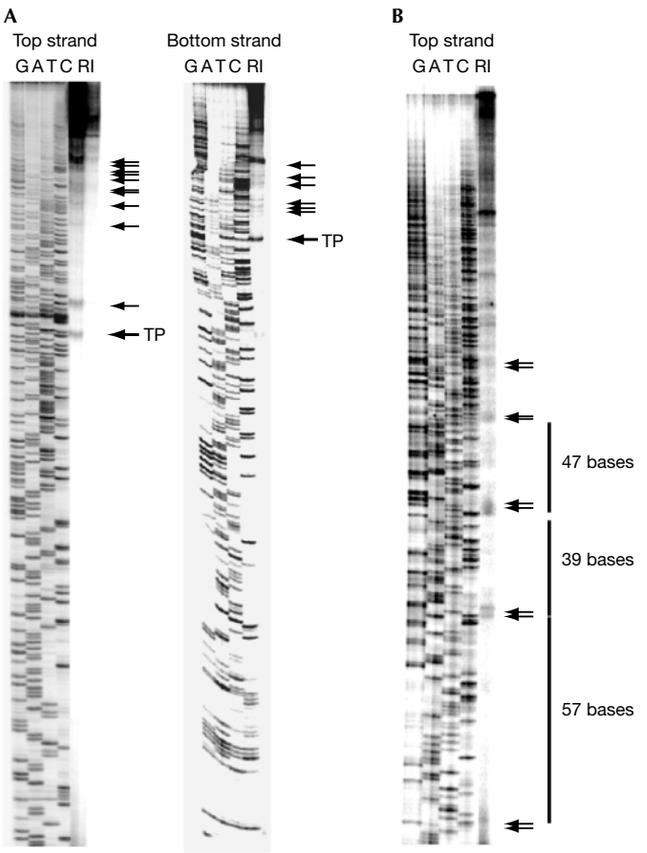

Fig. 3 | RIP mapping (for a detailed principle of the assay see the recent review by Bielinsky & Gerbi (2001)) revealed the frequent initiation of Okazaki fragments in the *P. abyssi oriC*. (**A**) RIP mapping of the 'top' and 'bottom' strands with the F1 or R2 primer (see Fig. 4A), respectively. Arrows indicate the transition point (TP). (**B**) Discontinuous replication on the top strand was analysed with the F2 primer. Sequencing reactions were run side by side on a denaturing polyacrylamide gel to map initiation points. RI, replication intermediates.

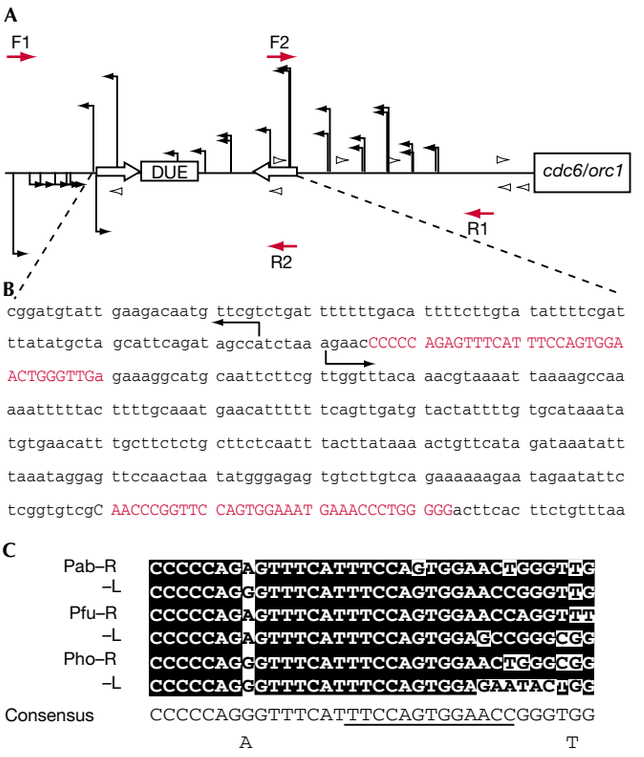

Fig. 4 | Detailed structure of the *P. abyssi OriC*. (**A**) Summary of initiation sites for DNA synthesis detected by RIP mapping. Stronger bands on the gels are shown by longer black arrows, and weaker bands by shorter black arrows. The long inverted-repeat and short 13-bp repeats are shown by open arrows and open arrowheads, respectively, together with the *cdc6/orc1* gene and a DNA-unwinding element (DUE). Primers used for RIP mapping of the top strand (F1 and F2) and the bottom strand (R1 and R2) are also indicated (red arrows). (**B**) Nucleotide sequence of a region containing the transition points (shown by arrows). The long inverted-repeat is shown in red capitals. (**C**) Conserved long inverted-repeats in *Pyrococcus* species. The consensus sequence is indicated at the bottom. Pab, *P. abyssi*; Pfu, *P. furiosus*; Pho, *P. horikosii*.

1.8% caffeine. About 10% of the DNA loaded was recovered by purification on the BND-cellulose column. Whenever possible, DNA samples were kept on ice in the presence of a relatively high salt concentration to stabilize DNA replication intermediates.

Labelling the 5′ termini of replication intermediates by an unmasking assay. The unmasking assay was performed essentially as described (DePamphilis, 1995). Purified total DNA was denatured for 2 min at 100 °C in 10 mM Tris-HCl, pH 8.3, and 0.1 mM EDTA, followed by rapid quenching in ice water at 0 °C. All 5′-OH termini existing in denatured DNA were first 'masked' by treatment for 1 h with 8 units of T4 polynucleotide kinase (PNK; Promega) and non-radioactive ATP at 37 °C in the buffer supplied by the manufacturer. RNA primers present on the 5′ ends of replication intermediates were hydrolysed (unmasking) by alkaline treatment for 16 h in 0.15 M NaOH and 1 mM EDTA at 37 °C. Exposed 5′-OH ends were labelled with PNK and [γ-^{32}P]ATP (5,000 Ci mmol^{-1}) at 4 °C and samples were run on an alkaline 1.8% agarose gel in running buffer (30 mM NaOH and 1 mM EDTA). Gels were dried on Whatman DE81 paper and were analysed with a Storm imaging system (Molecular Dynamics). The size marker (100-base ladder; Promega) was labelled by PNK with [γ-^{32}P]ATP.

Direct labelling of 5′ termini of RNA-primed replication intermediates by a capping assay. Purified total DNA was denatured as described above and treated with 6 units of guanylyltransferase (Gibco-BRL; note that the enzyme is no longer available from this source) in buffer containing 50 mM Tris-HCl, pH 7.9, 1.25 mM $MgCl_2$, 6 mM KCl, 2.5 mM dithiothreitol and 10 μM [α-^{32}P]GTP (5,000 Ci mmol^{-1}). After incubation for 1 h at 37 °C, labelled samples were fractionated on a denaturing 6% polyacrylamide gel and analysed by the Storm imaging system. Label was quantitatively removed by alkaline treatment, confirming the direct labelling of RNA.

RIP mapping. After phosphorylation of any 5′-OH ends with PNK, replication intermediates enriched on the BND-cellulose column were treated with λ-exonuclease to digest nicked DNA. Replication intermediates (20 μg) were incubated overnight at 37 °C with 7 units of λ-exonuclease (Gibco-BRL) in buffer containing 67 mM glycine-HCl, pH 8.8, 2.5 mM $MgCl_2$ and 50 μg ml^{-1} BSA. Digestion was confirmed on the agarose gel before proceeding to the primer extension reaction. For primer extension, ~500 ng of template DNA, 25 ng of the radiolabelled primer and 2 units of Vent (exo-) DNA polymerase were in the buffer provided by the manufacturer (New England Biolabs). After 30 cycles of primer extension reaction (1 min at 94 °C, 1 min at 70 °C and 1.5 min at 72 °C), samples were purified and analysed on a denaturing 6% polyacrylamide gel. The plasmid carrying the *P. abyssi oriC* on pBluescript II KS+ (Stratagene) was sequenced by Sequenase version 2.0 (from USB) with same primers as used for RIP assays. Sequencing and RIP reactions were analysed side by side on the same gel. Primers were designed with an online program, Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), to meet the following criteria (sequences are available from the authors on request): length 23–27 nucleotides; GC content >40%; T_m >68 °C. Primers were labelled by standard procedures using PNK with [γ-^{32}P]ATP (5,000 Ci mmol^{-1}).

ACKNOWLEDGEMENTS

H.M. and F.M. thank EMBO and the Japan Society for the Promotion of Science for financial support. H.M. is currently supported by CNRS. This work was supported by the Association pour la Recherche sur le Cancer (ARC), and the Programme de Recherches Fondamentales en Microbiologie, Maladies Infectieuses et Parasitologie (PRFMMIP) of the French Ministry of Science and Education.

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